IMMUNOLOGIC STUDIES ON HODGKIN'S DISEASE *

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In 1910 Fraenkel and Much[1] demonstrated a peculiar granular gram-positive bacillus in stained sections of lymph glands from Hodgkin's disease. In 1912 he[2] announced the demonstration of this organism in over 30 cases. All efforts at cultivating the organism, however, had been futile.

Negri and Mieremet[3] were the first to announce the isolation of the organism. Because of its peculiar morphology they classified it as a corynebacterium, and because of its association with Hodgkin's disease, as Corynebacterium granulomatis maligni. An attempt in two cases to show the presence of complement-fixation antibodies and agglutinins failed. The results of inoculation of the organism into animals were indefinite.

In 1913 Bunting and Yates[4] described the isolation of a pleomorphic diphtheroid organism from the lymph glands in 4 of 7 cases of Hodgkins' disease. In 1914[5] Bunting concluded that "Hodgkin's disease is an infectious disease due to a diphtheroid organism, the Bacterium hodgkini." Following their cultivation of the organism, they[1] inoculated a rhesus monkey repeatedly with 24-hour-old cultures of this bacillus. After 3 months, tissues excised from some of the enlarged lymph glands revealed histologic changes similar to those of glands early in Hodgkin's disease; namely, a chronic lymphadenitis with typical proliferation of endothelial cells, a beginning proliferation of the stroma, and a well-marked eosinophilic infiltration with a periglandular sclerosis.

Subsequently, Billings and Rosenow[6] confirmed these cultural results and isolated the organism from the blood in a few febrile cases. They also added the use of autogenous vaccine to the therapeutic

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[1] Ztschr. f. Hyg. u. Infektionskrankh., 1910, 67, p. 159.
[2] Deutsch. med. Wchnschr., 1912, 14, p. 637.
[3] Centralbl. f. Bakteriol, I, O., 1913, 68, p. 292.
[4] Arch. Int. Med., 1913, 12, p. 236. Jour. Am. Med. Assn., 1913, 61, p. 1803.
[5] Bull. Johns Hopkins Hosp., 1914, 25, p. 177.
[6] Jour. Am. Med. Assn., 1913, 61, p. 2122.

measures employed in the treatment of this disease. This led directly to the question of treatment with an immune serum.

At Dr. Billings' suggestion I undertook to ascertain whether an immune serum could be produced which would have either palliative or curative action in this disease. In April, 1914, immunization of a horse was commenced with cultures of organisms from the lymph glands in Hodgkin's disease. The Memorial Institute for Infectious Diseases afforded the facilities for the production of the immune serum, and Dr. P. G. Heinemann gave advice and assistance in the concentration of the serum.

IMMUNIZATION OF A HORSE AGAINST THE DIPHTHEROID BACILLUS OF HODGKIN'S DISEASE

Sixteen strains of diphtheroid organisms obtained from cases of Hodgkin's disease by Dr. E. C. Rosenow and Dr. Garde, were employed in the inoculation of the horse. Killed bacilli equivalent in amount to the 24-hour growths on 2 Loeffler's serum slants were injected subcutaneously. (The bacilli had been killed by heating for 1 hour at 60 C.) This dose produced a mild reaction in the horse, the temperature running up to 101 F. on the evening of the day following the vaccination. A 2nd dose of 10 slants of killed bacilli was given subcutaneously a week later, followed in a week by a 3rd dose consisting of 15 slants. Mild reactions were produced by these injections. A 4th dose of 20 c.c. of dead organisms—approximately 6 billions of bacteria to each cubic centimeter—was injected intravenously in the 4th week.

The needle of the syringe had been withdrawn from the vein but a few seconds when the horse began snorting, pawing, and throwing back its head, making at the same time several attempts to jump over the stall. Collapse followed, with slow difficult respiration, trembling of the legs with inability to stand on them, drooping of the head, and marked general weakness, the animal being held up by a supporting harness. At the end of 3 minutes the horse was trembling violently and drops of sweat had appeared over the entire body. Gradually strength returned, so that the animal could stand alone, and the shaking tremors diminished altho sweating increased, the sweat dropping continuously from the belly. The horse, still weak, was returned to the barn after 30 minutes. That evening its temperature was 103 F., a rise of 4.4 degrees since morning. The next day the animal was much stronger, the temperature (Chart 1) being practically normal. I have discussed this matter in detail as it is undoubtedly an anaphy-

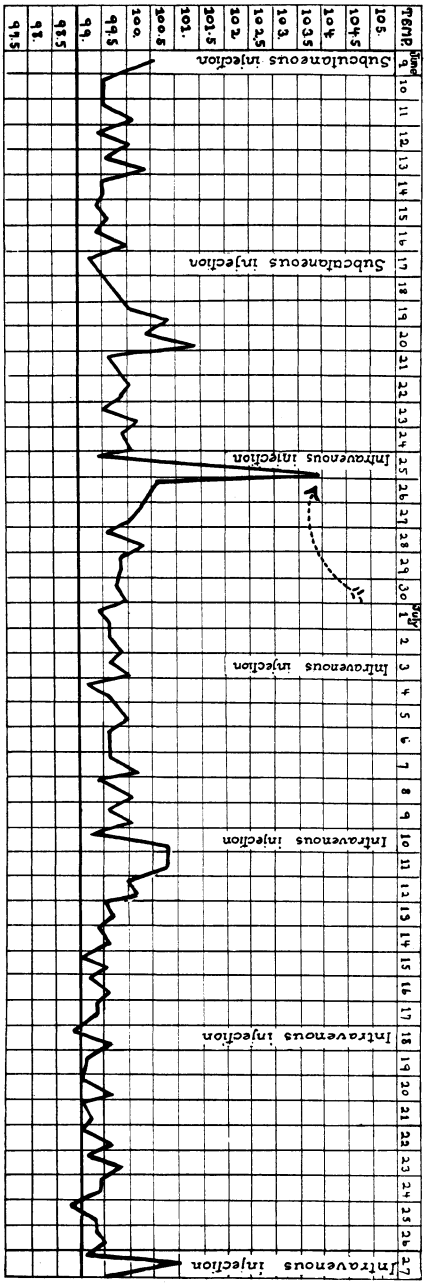

CHART 1

TEMPERATURE CURVE OF HORSE SHOWING RISE AFTER FIRST INTRAVENOUS INJECTION OF DIPHTHEROID BACILLI

lactic reaction in a horse. The rapidity of onset, respiratory symptoms, collapse, and recovery are similar to those described by Nichol[7] and others as allergic reactions in human cases. I find in the literature no similar case of anaphylaxis in a horse.

Subsequent doses of 20 and 25 c.c. of dead bacilli and of from 20 to 30 c.c. of living organisms produced at most very mild reactions. After 3 intravenous injections of dead bacilli, live organisms were employed. The routine dose finally adopted was 30 c.c. of the mixed living cultures. These organisms were grown for from 24 to 72 hours on Loeffler's serum agar, were then suspended in sterile salt solution, and centrifugated to throw down the coarse particles. Each cubic centimeter averaged from 3 to 4 billions of live bacilli. Injections were made intravenously from every 7 to every 10 days.

Except that described, the reactions were mild, consisting of a rise in temperature of from 0.5 degree to 2 degrees. The effects produced by both dead and living bacilli were the same. Chart 1 illustrates the temperature curve of the horse, showing the high rise in temperature after the 1st intravenous dose and the slight rise following the subsequent injections. In a previous paper by the author,[8] describing the production of antistreptococcic serum in horses, it was shown that, altho relatively small doses of killed streptococci were injected subcutaneously, the temperature ascended from 1 to 5 degrees after each injection. One is therefore led to conclude that the diphtheroid bacillus of Hodgkin's disease is of a low degree of virulence. Experiments on other species of animals give further support to this observation.

When the serum showed a high degree of potency, the horse was bled, 2 gallons being obtained each time. Bleedings were made every 3 or 4 weeks. The major portion of the blood serum was then refined by a modification of Banzhaf's method, which is similar to that employed in the refining of diphtheria antitoxin. The plasma is diluted with one-half its volume of water and enough saturated ammonium-sulfate solution added to make a saturation of 30%. It is then gently heated for 2 hours, being brought gradually to an end temperature of 60 C. While the precipitate is warm, it is filtered off rapidly and extracted with saturated NaCl solution. "The filtrate is measured and enough saturated ammonium-sulfate solution added to bring the saturation to 54%. The precipitate is then filtered off, pressed out to remove as much liquid as possible, and finally dialyzed for from 7 to 9 days, or until practically all the ammonium sulfate is removed.

"The salt-solution extract of the first precipitate is thrown down with acetic acid, the precipitate pressed out, and the NaCl and ammonium sulfate dialyzed out. This precipitate must be neutralized with sodium carbonate. The dialyzed globulin solutions are sterilized by filtration through Berkefeld filters. Tricresol (0.3%) is used as a preservative.[9]"

Various tests for determining the potency, or the antibody content, of immune sera are in use, each one depending on the specific antibody formed in greatest proportion, and usually, therefore, only one test is applied in a given instance. For example, in the determination of

[7] Jour. Am. Med. Assn., 1914, 63, p. 2225.
[8] Jour. Infect. Dis., 1914, 15, p. 215.
[9] Am. Jour. Pub. Health, 1912, 2, p. 43.

the value of antidiphtheric or antitetanic serum the antitoxin content is found; in the case of antityphoid serum the agglutinin value; in that of antistreptococcic serum the opsonic value; and in that of the antiserum for Rocky Mountain spotted fever[10] the potency titer is the amount of immune serum that will protect against 100 minimal lethal doses of the virus. The tendency of the organism isolated from Hodgkin's disease to clump and resist separation prevented accurate opsonic estimations. Overlooking this difficulty, however, I found but slight increase in opsonins in the immune serum as compared with that from a normal horse. Again, the organism is of such low virulence that it causes no constant acute condition in lower animals which we can inhibit or cure by a fairly definite quantity of serum. Since complement-fixation with bacterial antigens has been successful in the case of gonorrhea, pertussis, and typhoid fever, I eventually employed it in testing the immune serum.

MATERIALS AND TECHNIC OF COMPLEMENT-FIXATION TESTS

Antigens.—The following cultures were used in preparing antigens: 16 strains of diphtheroid bacilli isolated from patients with Hodgkin's disease and lymphosarcoma; 4 strains each of Staphylococcus albus and Staph. aureus; 8 strains of streptococci isolated from tonsils and lymph glands in chronic arthritis; 1 strain of a diphtheroid organism isolated from a lymph gland in chronic arthritis; and 2 strains of B. diphtheriae.

The antigens were prepared as follows:

Antigen 1. The strains of diphtheroid bacilli from Hodgkin's disease were grown on Loeffler's serum agar for 24 hours and then were suspended in sterile normal salt solution. This was centrifugated, the supernatant fluid poured off, the sediment mixed with 15 c.c. sterile salt solution, centrifugated again, the supernatant fluid discarded, and the sediment mixed with 15 c.c. sterile normal salt solution to which 0.5% phenol had been added as a preservative.

Antigen 2. Strains of diphtheroid bacilli grown for from 4 to 8 weeks on serum agar were prepared in the same way..

Antigen 3. A mixture of strains of diphtheroid bacilli grown for 24 hours and for from 2 to 8 weeks was prepared like Antigen 1.

Antigens 4, 5, and 6. These, corresponding to Antigens 1, 2, and 3, were washed and suspended as in the case of Antigen 1, then heated for 1 hour at 60 C.

Antigens 7 and 8. Young and old cultures of 2 strains were prepared as in the case of Antigen 1, as homologous antigens for the patients from whom these strains had been obtained.

Antigen 9. Staphylococcus albus (4 strains) and Staph. aureus (4 strains).

Antigen 10. Streptococcus hemolyticus and S. viridans (8 strains).

Antigen 11. Diphtheroid bacilli (1 strain).

Antigen 12. Bacillus diphtheriae (2 strains).

Antigen 13. Gonococci.

Antigen 14. Syphilitic antigen.

Controls for all antigens, except 13 and 14, were prepared by growing cultures of the respective organisms for from 1 to 4 days on blood-agar slants,

[10] Heinemann and Moore: Jour. Infect. Dis., 1912, 10, p. 294.

washing off the growths with sterile normal salt solution, centrifugating and washing the sediment twice, and then suspending the washed sediment in normal salt solution to which 0.5% phenol had been added as a preservative.

Hemolytic System.—The antichicken hemolytic system was used in all my tests. Washed chicken corpuscles were made up to a 2.5% suspension and used in doses of 1 c.c.; these corpuscles had been sensitized by the addition of twice the hemolytic dose of the hemolysin as found by previous titration. Fresh guinea-pig sera diluted to 8% were used as complement. The complement was titrated each day before the actual tests, and twice the hemolytic dose used with human sera, 3 times it with horse serum. In a recent communication Kolmer[11] has demonstrated that the chicken hemolytic system is more delicate than many others, as no natural amboceptors for chicken corpuscles are found in human sera in the amounts employed in the complement-fixation tests.

All sera were heated to 56 C. for 30 minutes and used in different amounts. Antigen, serum, and complement were incubated for 1 hour at 38 C., the sensitized corpuscles added, the whole again incubated for 1 hour, and then placed in the ice-box over night. Readings were made at the end of the hour and the next morning.

Antigen Titrations.—(1) The anticomplementary dose of each antigen was determined and one-quarter of this used as the antigenic dose. (2) The antigens were titrated with sera of different animals which were supposedly normal and one-quarter of the anticomplementary dose used. By the second method I observed that horse blood and rabbit blood contained more complement-binding substances than did human blood and monkey blood. In order to overcome this increased action of horse and rabbit blood and at the same time have a uniform antigenic dose I increased the quantity of complement to 3 times its hemolytic dose when using horse and rabbit sera. The antigenic doses of both methods then closely paralleled each other. In the sera of some of the rabbits, however, anticomplementary bodies were found in such large quantities that the animals had to be discarded. A 3rd method, that of titration with an immune serum, a method similar to that in use in syphilitic antigenic determinations, was not possible with all antigens in our earlier experiments. By repeated titrations the anticomplementary doses of the antigens were found to remain constant.

The adoption of one-quarter of the anticomplementary dose, in view of the fact that complement was increased to 3 times its hemolytic dose with certain sera in order to overcome all inherent anticomplementary bodies, reduced to a minimum the danger of nonspecific reactions.

In a comparison of the values of various gonococcal antigens Kolmer and Brown[12] assert that their best results were secured with a simple antigen composed of gonococci in sterile normal salt solution plus preservative. In this antigen the endotoxin and the bacterial protein, both of which add to the antigenic properties, are preserved. Since my desire was to obtain a good antigen and not to test various antigens, I adopted that method of preparing antigens which produced best results in gonorrheal complement-fixation. On the other hand, Olitsky[13] in a series of complement-fixation tests with the Corynebacterium hodgkini discarded antigens composed of unfiltered suspensions of the organism as being too anticomplementary and nonantigenic, and used only the filtrates of such suspensions. Since his results differ in no respect from mine, we may assume that both filtered and unfiltered antigens are satisfactory.

[11] Jour. Infect. Dis., 1915, 16, p. 441.
[12] Ibid., 1914, 15, p. 6.
[13] Jour. Am. Med. Assn., 1915, 64, p. 1134.

STANDARDIZATION OF IMMUNE HORSE SERUM

The first serum tested was withdrawn from the horse after the second injection of vaccine. Apparently few antibodies had been developed, as 0.2 c.c. of the serum did not inhibit hemolysis. Titrations were continued at irregular intervals. In September 0.1 c.c. serum caused complement-fixation with 0.04 c.c. antigen. I finally obtained inhibition of hemolysis with 0.0005 c.c. serum plus 0.1 c.c. antigen plus 3 times the hemolytic titer of the complement. Table 1 illustrates the high potency of the serum as determined by the complement-fixation method. The titer has remained at this point for several months.

TABLE 1

TITRATION OF HORSE SERA WITH DIPHTHEROID ANTIGEN. ANTIGEN — 0.1 C.C. COMPLEMENT — 3 HEMOLYTIC DOSES

Dose of Serum	Horse 1 Immunized	Horses 50 and 58 Controls	Horse 69 Control	Horse 66 Control
0.0001	75% hemolyzed	Hemolysis complete	Hemolysis complete	Hemolysis complete
0.0003	50% hemolyzed	Hemolysis complete	Hemolysis complete	Hemolysis complete
0.0005	Hemolysis inhibited	Hemolysis complete	Hemolysis complete	Hemolysis complete
0.0008	Hemolysis inhibited	Hemolysis complete	Hemolysis complete	Hemolysis complete
0.001	Hemolysis inhibited	Hemolysis complete	Hemolysis complete	Hemolysis complete
0.05	Hemolysis inhibited	Hemolysis complete	Hemolysis complete	Hemolysis complete
0.1	Hemolysis inhibited	Hemolysis complete	50% hemolyzed	Hemolysis complete
0.2	Hemolysis inhibited	Hemolysis complete	Hemolysis inhibited	50% hemolyzed

TABLE 2

TITRATION OF HORSE SERA WITH VARIOUS ANTIGENS. ANTIGENIC DOSE — ¼ ANTICOMPLEMENTARY DOSE; COMPLEMENT — 3 HEMOLYTIC DOSES; SERA — 0.1 C.C.

	Diphtheroid Bacilli 1	Diphtheroid Bacilli 3	Staphylococci	Streptococci	Diphtheria Bacilli	Gonococci	Syphilitic Antigen
Immunized Horse 1	Hemolysis inhibited	Hemolysis inhibited	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete
Control Horses 50 and 58	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete

In Table 2 the action of control bacterial and lipoidal antigens with immune and control horse sera are shown, demonstrating that the tests

are specific for the organisms used in immunization. Hemolysis resulted in all the control antigens but was inhibited in the diphtheroid antigens when immune horse serum was added. On the other hand, when sera from control horses were mixed with the diphtheroid antigen, hemolysis resulted. Over 25 titrations of the immune horse serum were made with marked inhibition of hemolysis in the higher dilutions which seemed evidence enough that I had produced an antiserum against the diphtheroid organisms isolated in Hodgkin's disease.

This unrefined antiserum was used in the treatment of a few cases of Hodgkin's disease, but the serum reaction was of such a severe nature, even with small doses, that concentration of the serum was immediately suggested. This was accomplished by the technic described. By this method the major portion of the proteins in the horse blood is eliminated and the antibodies are so concentrated that much smaller doses can be administered. I encountered a difficulty, however, in measuring the potency of the refined serum. Concentration had so increased the anticomplementary action that titration by the complement-fixation method was of little value. In amounts of 0.01 c.c. the refined serum was anticomplementary, but not in doses smaller than 0.0005 c.c., the same titer as the unrefined serum. Measured solely by the complement-fixation test, there would apparently be little gain in refining the serum.

Agglutination tests were then undertaken.

Cultures were grown for 24 hours on Loeffler's serum agar, then washed off with sterile salt solution, emulsified, and diluted with sterile salt solution to a satisfactory cloudiness. All tests were made by the macroscopic method. The serum was used in the dilutions shown in Table 3. Five-tenths cubic centimeter serum-dilution plus 0.5 c.c. bacterial emulsion was added to small test tubes, which were then incubated for 2 hours at 37 C., placed in the ice-box for 12 hours, and afterward read. Each set also included a control tube of 0.5 c.c. normal salt solution plus 0.5 c.c. bacterial emulsion.

In 4 tests the control serum caused agglutination at 1 : 640 dilution, the unrefined immune serum at 1 : 2560 dilution, and the refined immune serum at 1 : 5120 dilution. The unrefined immune serum contained approximately 4 times more agglutinin than normal horse serum, and the refined approximately twice as much as the unrefined. While some complement-binding antibodies were apparently destroyed by the process of refining, the agglutinin did not undergo such a marked change.

Table 3 illustrates the intensity of the agglutination reaction in the testing of the sera. While these experiments do not indicate the therapeutic value of the serum, they show that there is immunization produced against this organism with a probable formation of curative antibodies.

More important even than the concentration of the antibodies was the diminution in the nonspecific allergic reactions with the use of the refined serum. Much larger doses of refined serum could be administered to patients than of unrefined, with less of the distressing symptoms of serum disease.

TABLE 3

AGGLUTINATION WITH HORSE SERA

Dilution	Unrefined Immune Serum	Refined Immune Serum	Control Serum
1:20	Marked	Marked	Distinct
1:40	Marked	Marked	Slight
1:80	Marked	Marked	Slight
1:160	Marked	Marked	Slight
1:320	Distinct	Distinct	Slight
1:640	Slight	Distinct	Slight
1:1280	Slight	Slight	None
1:2560	Slight	Slight	None
1:5120	None	Slight	None
1:10240	None	None	None

The opsonic value of the serum, measured by the opsonic index, proved to be negative. The index of the immune horse was no greater than that of the control horses. Similar results were obtained by Dr. Garde and Dr. Coleman with sera from patients with Hodgkin's disease, altho in some instances these patients had received numerous doses of vaccine.

EXPERIMENTS ON MONKEYS

In the summer of 1914 I attempted to produce Hodgkin's disease in a rhesus monkey by repeated injections of mixed cultures of the living bacilli which I had at hand, isolated from human cases of the disease. The following report for Monkey 1 gives this experiment in condensed form:

Monkey 1.—Macacus rhesus, male. On July 24, 1914, the animal was injected subcutaneously in the left side of the neck with 1 c.c. of an emulsion containing 8 strains of living bacilli—about 2 billion organisms per cubic centimeter. No lymph glands palpable in axillae or cervical regions. Inguinal lymph nodes slightly enlarged. Leukocytes 12,500. Temperature 101.7.

August 1.—Injected subcutaneously in left side of neck with 1 c.c. of a mixture of 9 strains of living bacilli. A lymph gland approximately 1 cm. in length could be palpated on the upper left side of the neck. Leukocytes 17,800. Erythrocytes 5800. Temperature 102.

August 8.—The same lymph gland still enlarged. Several smaller ones of the cervical chain could be felt. Injected in the same location with 2 c.c. of an emulsion of 13 strains—approximately 5 billion organisms per cubic centimeter. Leukocytes 12,500. Erythrocytes 5,287,000. Temperature 102.4.

August 15.—Injected with 2.5 c.c. of the mixed cultures of 12 strains. Glands of neck small. Leukocytes 14,800. Temperature 102.6.

August 22.—Injected with 2 c.c. of 12 mixed strains—about 7 billion organisms per cubic centimeter—in the cervical region. Glands of neck small. Palpable axillary glands on right side. Leukocytes 11,600. Temperature 102.6.

August 29.—Injected with 3 c.c. of 12 strains—7 billion organisms per cubic centimeter. Lymph glands on right side of neck showed no further enlargement. Monkey appeared well. Leukocytes 13,900. Temperature 102.2.

Injections were made of from 2.5 c.c. to 3 c.c. of 12 strains in the cervical region on Sept. 5; in the left axilla on August 12, Sept. 19, and 26, and Oct. 3; and in the left inguinal region on Oct. 17, with the production of one palpable gland in the left axilla. The enlarged cervical glands disappeared, as did those in the right axilla. The inguinal glands remained about the same. The left axillary glands became much smaller and were just palpable 3 weeks after the last injection. The leukocytic count varied between 12,000 and 17,000. The temperature did not go over 102.4. The monkey reacted very little towards these comparatively large injections. There was no induration around the glands, as described by Bunting and Yates,[4] the individual nodes being freely movable. Monkey 1 died 3 months after the last injection.

The leukocytic count in this monkey was somewhat similar to that of the monkey used by Bunting and Yates.[4] Their monkey from the beginning of the experiment had a higher leukocytic count, averaging 20,450 in 7 counts, while mine averaged 13,500 in 13 counts. As I gave larger and more frequent injections, a higher white count might have been expected but this did not prove to be the case in the actual experiment. Anderson and Neill[14] found the following blood picture as an average of 100 observations on 10 healthy rhesus monkeys of both sexes: Leukocytes 11,192; neutrophiles 46.61%; basophiles (lymphocytes and large mononuclears) 50.65%; eosinophiles, 3.69%; mast cells 0.24%; unclassified, transitionals, 0.8%. In but 1 monkey was the percentage of neutrophiles higher than that of mononuclears; in 1 it was the same; and in the remaining 8 the lymphocytic count was from 6 to 30% higher than that of the neutrophiles. If these counts may be regarded as a norm, there was apparently an increase in the neutrophiles with a corresponding decrease in the lymphocytes in Monkey 1. The percentage of eosinophiles was so irregular that I could not draw any conclusions.

Table 4 illustrates the individual counts and gives the average of Anderson's and Neill's normal counts, together with an average of the 12 counts made by Bunting and Yates. To facilitate counting I have included the large lymphocytes and transitionals under large mononuclears.

Monkey 2.—Injected intravenously with 10 c.c. of the mixed emulsion of 15 strains. It was ill for a few days. Fourteen days later 6 c.c. were given intravenously, after which it was ill for 2 days. Seven days later it was given 5 c.c. intramuscularly in the right thigh and 6 c.c. intravenously in the left femoral vein. It lost its appetite and died on the 5th day. Cultures from the heart blood and from the abscess which developed in the left thigh, contained diphtheroid and colon bacilli. No greatly enlarged glands were found,

[14] Jour. Med. Research, 1915, 33, p. 143.

the largest in the inguinal region of the left side being 7 mm. in size. Histologically, the glands were hyperplastic, as in an ordinary acute infection.

The white blood count in this monkey was high before the experiment, being 24,000, while the temperature was 104. Blood counts were not taken during the experiment.

This monkey died from septicemia due to contamination with B. coli, its death occurring too early for the clinical picture of Hodgkin's disease to develop. However, the 1st monkey showed nothing which clinically resembled Hodgkin's disease.

TABLE 4

BLOOD COUNTS OF MONKEY 1, COMPARED WITH ANDERSON AND NEILL'S NORMAL AVERAGE AND BUNTING AND YATE'S AVERAGE

Date	Leukocytes	Neutrophiles	Small Lymphocytes	Large Mononuclears	Eosinophiles	Mast Cells
7/24	12,500	49.6	43.4	2.7	4.3	.0
8/15	14,800	51.0	42.0	4.8	2.0	.2
8/29	13,900	59.2	35.0	4.6	1.0	.2
9/ 5	12,000	59.0	32.5	7.1	1.1	.3
9/12	12,500	68.8	22.5	5.1	3.6	.0
9/19	12,000	53.4	40.6	3.1	2.6	.3
9/26	17,000	59.0	31.3	6.3	3.3	.1
10/ 3	13,500	55.5	38.5	5.5	0.5	.0
10/ 7	12,600	52.2	39.0	7.8	1.4	.4
10/24	13,200	61.3	29.4	3.0	6.7	.2
Average.....	13,500	56.9	35.47	4.79	2.75	.17
Anderson and Neill's average.....	11,192	41.61	54.45		3.69	.24
Bunting and Yate's average.....	20,450	46.0	40.9	10.73	2.6	.35

The technic previously described for the standardization of the horse-serum complement-fixation tests, employed for the monkeys gave somewhat parallel results. After the series of injections the serum of Monkey 1 fixed complement when 0.05 c.c. of serum was used. Before the experiment hemolysis had occurred with 0.2 c.c. of serum. In the case of Monkey 2 serum from blood drawn at the time of the last injection prevented hemolysis in doses of 0.005 c.c. Before the experiment 0.3 c.c. had failed to prevent hemolysis. Agglutination and opsonic tests were not tried with the sera of the monkeys.

EXPERIMENTS ON RABBITS

Before the inoculation of rabbits was commenced, complement-fixation tests were made on 5 animals, the sera of 2 of which fixed complement in doses as small as 0.05 c.c., of 1 in dose of 0.1 c.c., while the sera of the remaining 2 gave complete hemolysis in doses of 0.2 c.c.

The latter animals were used for immunization. The phenomenon of complement-binding when normal rabbit serum is added to bacterial antigens is well known. Olitsky[13] in testing a series of rabbits before immunization with Hodgkin's bacilli obtained the same result and discarded animals which bound complement in high dilutions in the presence of antigen.

Immune rabbit sera were prepared as follows: 4 billion organisms of a mixed emulsion of live bacilli were injected subcutaneously; followed in 5 days by 10 billions, then by 5 doses increasing from 4 to 10 billions, intravenously every 5 days. Serum was withdrawn at the end of 10 days and found to bind complement completely in doses of 0.0003 c.c. The organism appeared to be entirely avirulent for rabbits, but produced a high degree of immunity as measured by complement-fixation.

TOXIN-PRODUCTION

Two Erlenmeyer flasks of glucose broth were each inoculated with 12 strains of diphtheroid bacilli isolated from Hodgkin's disease. One was incubated for 5 days, the other for 10 days. At the end of these periods both were centrifugated for 20 minutes at 3400 revolutions a minute, and then 5 c.c. of the supernatant broth injected into the peritoneal cavities of two 250-gram guinea-pigs. As neither of the guinea-pigs appeared in the least discomfited by the large amounts of broth, we may judge that soluble toxin in appreciable amounts was not produced. These findings agree with the results obtained by others[15, 16, 17] as to toxin-production by diphtheroid organisms.

COMPLEMENT-FIXATION IN PATIENTS WITH HODGKIN'S DISEASE

In this series of studies I was fortunate enough to have the sera from 10 cases (9 of which were in Dr. Billings' service) diagnosed as Hodgkin's disease. In 9 of these, histologic examination of lymph glands confirmed the clinical diagnosis and cultures from the majority of the cases resulted in the growth of a pleomorphic diphtheroid organism.

The technic in these tests was the same as that described. From 0.1 to 0.6 c.c. of the patient's serum was used, the usual amount being from 0.1 to 0.2 c.c. Sera were obtained from the following cases of Hodgkin's disease. (The histories are not complete, giving only the

[15] Hamilton: Jour. Infect. Dis., 1904, 1, p. 690.
[16] Clark: Jour. Infect. Dis., 1910, 7, p. 335.
[17] Fox: Jour. Med. Research, 1915, 32, p. 325.

data which are related to the tests. All treatments other than with vaccine and serum are omitted.)

Case 1.—Clinical diagnosis, Hodgkin's disease. Cervical glands first enlarged 2 years before first test. Nine months after appearance of symptoms a gland had been removed and diagnosed as Hodgkin's disease by the Mayo Clinic. Diphtheroid bacillus had been isolated from a gland removed at the Presbyterian Hospital, Chicago, and this made into an autogenous vaccine. Histologic examination of the gland had confirmed the previous diagnosis. Patient had had 14 doses of vaccine before the first complement-fixation test. The 6 tests covered a period of 5 months. Previous to the last test the patient had received over 50 doses of nonsensitized and sensitized autogenous vaccine, ranging from 10 million to 1 billion organisms each. With this were given several doses of immune horse serum. The patient left the hospital much improved, having gained 19 pounds in weight.

Case 2.—Clinical diagnosis, Hodgkin's disease. Pathologic diagnosis of excised lymph gland, Hodgkin's disease. The organism isolated from the gland was a coccus form. Duration of disease about 1 year. Seven doses of a mixed Hodgkin's and staphylococcus vaccine had been administered before the first test, 15 before the last, together with several doses of immune horse serum. Some improvement noted. The tests covered a period of about 2 months.

Case 3.—Clinical diagnosis, Hodgkin's disease. The symptoms had been present over 2 years. A pleomorphic diphtheroid organism had been isolated from an excised lymph gland and at the same time a histologic diagnosis of Hodgkin's disease had been made. Over 12 vaccinations had been made before the first test, and 9 before the last test. Three tests were made in a period of 6 weeks. The patient died a week after the last test.

Case 4.—Clinical diagnosis, Hodgkin's disease. The first symptom had appeared over 2 years before. Cultures from excised lymph glands had been sterile. Histologic diagnosis, Hodgkin's disease. No vaccine had been given before the first test, but over 40 doses of mixed diphtheroid bacilli ranging from 1 to 5 million organisms each, in addition to immune horse sera, had been injected before the last test. The 5 tests covered a period of 3 months.

Case 5.—Clinical diagnosis, Hodgkin's disease. The symptoms had lasted 18 months before the first serum was obtained and about 20 vaccinations had been made. The histologic diagnosis had been Hodgkin's disease and a diphtheroid organism had been isolated from the lymph glands. Over 50 injections of nonsensitized and sensitized autogenous vaccine with immune horse serum had been administered previous to the last of 6 tests. The serum was examined once a month for 5 months. In this case I used not only the mixed antigens but an antigen prepared from the bacillus isolated from the patient's diseased lymph gland. The patient received 1 dose of vaccine intravenously. Agglutination tests were also made on this serum.

Case 6.—Clinical diagnosis, Hodgkin's disease. The first symptoms had been noted 6 months before the test. A diphtheroid organism had been twice isolated from the lymph glands. One dose of vaccine had been injected before the test. The patient died.

Case 7.—Clinical and histologic diagnosis, Hodgkin's disease. The symptoms had persisted for 3 years. No vaccine therapy had been employed previous to the first serum examination. Nine doses of autogenous vaccine were given before the last test.

Case 8.—Clinical and histologic diagnosis, Hodgkin's disease. A diphtheroid bacillus had been isolated from an excised lymph gland. No vaccination had been made previous to the first test. An antigen prepared from the organism isolated from the patient was also used. Tests for agglutinin were also made on this serum.

Case 9.—Clinical and pathologic diagnosis, Hodgkin's disease and congenital lues. The glands had been enlarged for 18 months. The test was made before any vaccine had been given. A positive Wassermann was repeatedly obtained with this serum.

Case 10.—Clinical diagnosis, Hodgkin's disease. The symptoms had been obvious for over 18 months. No vaccine had been given before the one test. The glands have not yet been examined histologically.

The sera from several cases diagnosed clinically as Hodgkin's disease, but which microscopic examination of glands proved to be lymphosarcoma or tuberculosis, were tested. One of these, a lymphosarcoma, requires special consideration, as a pleomorphic diphtheroid bacillus morphologically and culturally identical with those obtained from the Hodgkin's glands was isolated from the glands.

Case 11.—Clinical diagnosis, Hodgkin's disease. Histologic diagnosis, lymphosarcoma of cervical glands. The condition had been progressing for 11 months. The patient had had several doses of autogenous vaccine before the first complement-fixation test and over 40 before the last. Immune horse serum was also employed. The tests extended over 2 months. The patient has since died. This case may be classed immunologically with the cases of Hodgkin's disease.

The complement-fixation tests were made at irregular intervals over a period of more than 5 months in 2 cases; 3 months in 1 case; 2 months in 1 case; 1 month in 3 cases; and examination was made but once in 3 cases—in all, a total of 31 different examinations. In no instance did I find inhibition of hemolysis with any of the various diphtheroid antigens or with any of the control antigens except in the case of the Wassermann reactions (Case 10). Olitsky[13] obtained similar negative results in a series of 6 cases.

In Cases 5 and 8 an antigen was prepared from the organisms isolated from the patients and the patients' sera tested against these homologous antigens. The reactions were negative in both cases.

Apparently no complement-binding antibodies had been produced by the vaccinations. Three individuals were tested before they had received any vaccine, and 1 after but 1 dose. Others had had from 7 to 10 doses before the first test and some over 50 before the last test. One dose of vaccine contained from 10 million to 1 billion organisms. One patient had a dose of vaccine intravenously which produced a

severe general reaction, but tests failed to show any positive complement-fixations.

In Case 10 a positive Wassermann reaction was obtained several times in different laboratories. This case was diagnosed clinically as congenital lues plus Hodgkin's disease. The histologic diagnosis was Hodgkin's disease. As in all other cases there was complete hemolysis with the lipoidal antigens, we may assume that Hodgkin's disease and lues are not closely related. It is of interest that negative Wassermanns were obtained for the 8 cases of myelogenic and lymphatic leukemia, and for 2 cases of lymphosarcoma, as well as for all other serum controls except the syphilitics.

TABLE 5

COMPLEMENT-FIXATION TESTS IN HODGKIN'S DISEASE

No. of Cases	Diagnosis	No. of Tests	Result	Remarks
10	Hodgkin's disease.....	31	Hemolysis	Six cases had vaccine
2	Lymphosarcoma.....	6	Hemolysis	Diphtheroid bacillus isolated from 1 case
6	Myelogenic leukemia....	12	Hemolysis	
2	Lymphatic leukemia....	2	Hemolysis	
4	Tuberculosis.....	5	Hemolysis	Three cases of tuberculous lymphadenitis
2	Asthma.....	3	Hemolysis	
6	Syphilis.....	6	Hemolysis	
1	Chronic arthritis.....	1	Hemolysis	
1	Chronic arthritis.....	1	Hemolysis	
4	Normal.....	6	Hemolysis	
1	Immunized horse.....	25	Hemolysis inhibited	Injected with live bacilli for 6 months
5	Control horses.....	26	Hemolysis	Three diphtheria antitoxin horses were included

Since staphylococci had been isolated from the diseased glands in Hodgkin's disease,[6] it was thought possible that some of these sera might react positively with staphylococcus antigen. This was not found to be true.

As controls, sera from the following diseases (see Table 4) were tested with the several antigens: myelogenic leukemia, 6 cases; lymphatic leukemia, 2 cases; lymphosarcoma, 2 cases; tuberculous lymphadenitis, 3 cases; pulmonary tuberculosis, 1 case; asthma, 2 cases; chronic arthritis, 1 case; syphilis at various stages, 6 cases; chronic nephritis, 1 case; and normals, 4 cases. The cases of leukemia were selected because of the close relationship of that disease to Hodgkin's disease or pseudoleukemia, and in view of the isolation by Steele[18] and Simon and Judd[19] of diphtheroid organisms from lymphatic leukemia;

[18] Boston Med. and Surg. Jour., 1914, 170, p. 123.
[19] Jour. Am. Med. Assn., 1915, 64, pp. 20, 1630.

the cases of lymphosarcoma were selected for similar reasons; tuberculosis, because of the clinical resemblance of tuberculous lymphadenitis to Hodgkin's disease, and because similar organisms had been isolated in this disease; chronic arthritis, because diphtheroid organisms had been found in lymph glands in this condition; syphilis was selected for the purpose of ascertaining whether a positive syphilitic blood would react positively with the bacterial antigens used. The tests with homologous antigens were negative in every case.

AGGLUTINATION TESTS ON PATIENTS WITH HODGKIN'S DISEASE

The sera of Cases 5 and 8 were examined for agglutinin. In both cases bacterial emulsions consisting of organisms isolated from the patients as well as mixed emulsions of bacilli from other cases of Hodgkin's disease were tested. No higher agglutination could be observed in the patient's sera than in the normal controls, either with the homologous, or with the mixed emulsions. In a personal communication from Dr. Garde and Dr. Coleman I learn that they have made several agglutination tests on other pseudoleukemic patients using homologous antigens with negative results.

SUMMARY

Horses can be immunized by repeated intravenous injections of pleomorphic diphtheroid bacilli isolated from the lymph glands in Hodgkin's disease.

In complement-fixation tests this immune serum bound complement when used with these same organisms as antigens in amounts as small as 0.0005 c.c. Complete hemolysis occurred with control antigens of staphylococci, streptococci, diphtheria bacilli, gonococci, and lipoidal extracts and 0.1 c.c. of the immune serum. Complete hemolysis resulted when control horse sera were added to the diphtheroid antigen in amounts of 0.1 c.c.

Agglutinin was found to be increased fourfold by the immunization.

By refining this serum according to the methods employed in concentrating diphtheria antitoxin, it was found that altho no definite increase in the complement-fixing antibodies could be demonstrated, an increase in agglutinin could be shown. The refined serum produced fewer allergic reactions than the whole serum.

Monkeys were immunized with the same organisms by both subcutaneous and intravenous inoculations. An increase in complement-

binding amboceptors was found in the serum after the injections. By similar methods immune sera were obtained from rabbits.

Soluble toxins were not formed by these organisms when grown in glucose broth.

Complement-fixation tests were made on 10 individuals having Hodgkin's disease and in no case was there inhibition of hemolysis with antigens of the mixed cultures isolated from Hodgkin's disease. In 2 cases antigens prepared from organisms cultured from the patients' glands were used, with negative results.

Vaccination with these organisms did not appear to increase the complement-binding antibodies in patients.

Complement-fixation tests on the sera of these patients with control antigens were negative in all except one instance in which positive Wassermanns were repeatedly obtained.

Agglutination experiments with the sera of 2 patients were likewise negative.

Complement-fixation tests made on sera from cases of lymphosarcoma, lymphatic leukemia, chronic arthritis, and tuberculosis, diseases in which diphtheroid organisms have been isolated, all reacted negatively with the various antigens used.